

Expression of Pro-inflammatory Chemokines CXCL1 and CXCL5 is Rapidly Upregulated Following Traumatic Injury

Matthew Jergenson, Gengyun Le, PhD, Dawn A. Lowe, PhD

**Submitted under the supervision of Dr. Dawn A. Lowe, Department of Physical Medicine
and Rehabilitation, University of Minnesota- Twin Cities**

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II. Abstract

When a traumatic skeletal muscle injury occurs, such as crushing, contusion, laceration, or freezing, the body must mount an inflammatory response in order to clear muscle debris and fully regenerate the damaged muscle. This response is made up primarily of an innate immune response, which is amplified by the release of pro-inflammatory cytokines and chemokines and leads to the activation of several specific myeloid cell populations. The neutrophil, the first myeloid cell to infiltrate injured muscle, is attracted by the cytokines IFN- γ and TNF- α and the chemokines CXCL1 and CXCL5. However, the timing and magnitude of CXCL1 and CXCL5 signaling following muscle injury have not been carefully examined. Using a freeze injury model to induce a traumatic injury to muscle and real-time PCR, we showed that CXCL1 and CXCL5 expression is rapidly upregulated, within 60 minutes and 3 hours, respectively, in mouse tibialis anterior (TA) muscles following injury. This finding serves as a starting point in the characterization of pro-inflammatory chemokines' impacts on the muscle injury inflammatory response.

III. Introduction

The human body contains three types of muscle tissue: cardiac, smooth, and skeletal. Cardiac muscle, as the name implies, is found in the heart and powers the organ's repetitive cycles of contraction and relaxation as it pumps blood through the body. Smooth muscle is located in many areas of the body that are lined with an epithelium, such as the GI tract, and controls a multitude of essential body functions without conscious effort. And finally, skeletal muscle, the type of tissue most commonly associated with the term "muscle", is composed of force-generating units that support locomotion and allow us to do work. The interest of this study lies in the last type of muscle tissue.

Most skeletal muscles are located superficially in the body and are stressed daily by the many tasks that we ask them to perform, as well as by a multitude of unexpected physical

traumas, such as crushing, contusion, laceration, or freezing. These types of muscular injuries are common in humans and can have long-lasting effects on the muscle's integrity. When traumatic muscle injuries do occur, the tissue mounts an inflammatory response in order to fully regenerate the damaged muscle. In general, inflammation is simply the immune system's reaction to a stimulus, a damaged muscle in this case. Although often viewed as negative, inflammation is actually a necessary component of skeletal muscle's acute response to injury, as its processes are essential for the eventual restoration of the tissue's form and function (Tidball 2012). In order for the muscle to recover fully, damaged fibers must be broken down, removed, and replaced by previously undifferentiated cells called satellite cells (Summan 2006). Attenuation of the inflammatory response can result in prolonged clearance of necrotic (dead) myofibers and increased accumulation of fat in the muscle post-injury (Summan 2006). If the muscle heals improperly, its functional recovery can be incomplete, it can have a tendency to become injured again, and scar tissue may form (Huard et. al 2002), similar to what occurs in diseased muscle with chronic inflammation.

Skeletal muscle's inflammatory response to a sterile, traumatic injury is made up primarily of an innate immune response (Tidball 2012). Innate immunity is a protective system that is present at birth and does not have to be acquired through contact with a foreign body. It allows organisms to mount an immediate defense against invaders, but has no lasting memory of the attacker and treats all foreign bodies in much the same way. At the beginning of this innate response following a traumatic muscle injury, T-helper cells, cells that work to increase the activation of other immune cells, are activated in response to the damaging of the tissue. These T-helper cells then release a group of pro-inflammatory cytokines called Th1-type cytokines that help to amplify the immune signal. This signal produces a response by several specific myeloid

cell populations, which are activated and attracted to the site of the injured muscle (Tidball 2010). Myeloid cells are secretory cells that, when activated, release an array of soluble molecules that affect viability and transcriptional activity in regenerating muscle cells (Tidball 2010). The first of these populations, and the one that is relevant to this study, is the neutrophil, a phagocytotic (cell-engulfing) cell population that infiltrates the damaged muscle tissue and begins to break down cellular debris. The influx of neutrophils is caused by the cleavage of soluble proteins in the complement system following injury (Tidball 2012) as well as attraction by the pro-inflammatory cytokines IFN- γ and TNF- α , and the pro-inflammatory chemokines CXCL1 and CXCL5, all of which are released by activated T-helper cells (Tidball 2010).

Previously, levels of CXCL1 have been found to be significantly increased in mouse EDL and soleus muscles after just a 30 min treadmill protocol (Nedachi 2008). ELISA experiments by the same group found secretions of both CXCL1 and CXCL5 to be increased within 1 hour of electric pulse stimulation in vitro, and these increases were accompanied by rises in the concentrations of CXCL1 and CXCL5 mRNAs (Nedachi 2009). However, beyond these limited time point studies, the time at which these signals first appear and how long they persist following induction of a traumatic skeletal muscle injury is not known. This is important knowledge to our understanding of the impacts of CXCL1 and CXCL5 signaling and the chemokines' effects on the muscle injury inflammatory response overall.

Through the use of a freeze injury model and real-time polymerase chain reaction (qPCR), we evaluated the expression of the CXCL1 and CXCL5 genes at various time points after injury induction. Based on the work of the Nedachi group, I hypothesized that CXCL1 would be significantly upregulated 30 minutes post-injury and CXCL5 would be upregulated 1 hour after.

IV. Materials and Methods

Animals

Twenty-one female C57BL/6 (wildtype) mice were acquired from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of 3-5 and had access to phytoestrogen-free rodent chow (Harlan-Teklad; Indianapolis, IN) and water ad libitum. The room was maintained on a 12:12 light:dark cycle.

Experimental Design

A freeze injury was induced to the tibialis anterior (TA) muscles of twenty-one mice in the study, with the exception of a control group (3 mice). Then, at immediate, 30 minute, 1 hour, 3 hour, 5 hour, and 12 hour time points (n=3 per time point), the TA muscles were harvested for analysis. Thirty minutes were counted from the time that the steel probe was pressed to the TA muscle until the time that the lethal pentobarbital overdose was administered. The different recovery periods were used in an attempt to identify when inflammatory chemokine gene expression changes first occur and to what extent.

Freezing Injury and TA muscle excision

The mice were anesthetized via inhalation with 1.75% isoflurane mixed with oxygen at a flow rate of 200 ml/min. Body temperature was maintained using a water-re-circulating heatpad,

and the lateral and anterior surfaces of the hindlimbs were shaved and prepared aseptically using betadine solution and 70% ethanol. Each mouse received 0.1333 mg/kg body weight buprenorphine subcutaneously. Then, a 1.5 cm incision was made through the skin to expose the TA muscle. A steel probe with a rounded tip of 6 mm diameter was cooled to the temperature of dry ice for 30 minutes as previously described in the scientific literature (Warren 2004). The probe was applied to the muscle mid-belly for 10 seconds to induce a freeze injury. Mice in the immediate, 30 minute, and 1 hour groups remained under anesthesia until muscle harvest. For the mice in the 3 hour, 5 hour, and 12 hour groups, 6-0 silk suture and vetbond were used to close the incision, and the site was treated with hydrogen peroxide. Each mouse in these groups also received 0.1333 mg/kg body weight buprenorphine subcutaneously approximately 5 minutes after withdrawal from isoflurane anesthetic (Moran 2006).

For harvesting, mice were euthanized via intraperitoneal injection with a 200 mg/kg body weight overdose of pentobarbital sodium (Diamondback Drugs; Scottsdale, AZ) and both TA muscles were excised. TA muscles were flash-frozen in liquid nitrogen and put in the -80°C freezer for long-term storage. All protocols and animal care procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC).

RNA Isolation

TA muscle samples in 2 mL microcentrifuge tubes were removed from the -80°C freezer and placed on ice for 10 minutes to thaw. Each muscle was then placed into a small plastic Petri dish and cut into 4 or 5 pieces using a sterilized forceps and a razor blade. The muscle pieces were returned to their tubes and 1 mL of TRIzol reagent (Ambion by Life Technologies; Eugene, OR) was added to each tube. The TRIzol was kept on ice throughout the isolation protocol. A Tissue Tearer® (Biospec Products; Bartlesville, OK) was then used to homogenize each muscle

at high speed for 30 seconds. If small pieces could still be observed in the tube, the tissue was subjected to 15 more seconds of homogenization until muscle material was no longer visible. After each muscle, the Tissue Tearer® was cleaned by running it at high speed in a beaker of 10% bleach solution and 3 different beakers of distilled water. The homogenizer was run for 30 seconds in the the 10% bleach and two of the water beakers, and for 20 seconds in the third beaker of water.

After the muscle tissue was homogenized, RNA was isolated by following instructions in the commercially available RNeasy® Kit (Qiagen; Alameda, CA). The RNA was then quantified by placing 1.5 µL in the Nanodrop® ND-1000 spectrophotometer (Thermo Scientific; Waltham, MA) and recording the RNA concentrations (ng/µL). The ratio of spectrophotometric absorbance at 260 nm to that at 280 nm was used to assess RNA purity. A ratio range of 1.9-2.1 was desired for use in future cDNA synthesis and Real-time PCR (qPCR).

cDNA synthesis

cDNA synthesis was performed using a commercially available high-capacity RNA to cDNA kit (Applied Biosystems by Life Technologies; Eugene, OR). The kit contained oligonucleotides, cDNA master mix, and reverse transcriptase enzyme. 1 µg of RNA was used for each sample and the manufacturer's protocol was followed.

Real-time PCR (qPCR)

Two hundred nanograms cDNA from each sample were used in Taqman Fast-Advanced master mix (Life Technologies) to perform qPCR gene expression assays, following the manufacturer's protocol. The expression of the pro-inflammatory chemokines CXCL1 and CXCL5 was used to assess differences between the control and freeze-injured muscles. *Hprt* and

Hsp90ab1 (Life Technologies) were used as housekeeping genes to compare relative gene expression increases between the control and experimental groups. The comparative threshold (CT) method was used to calculate relative concentrations. Samples were assayed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistics

Differences in gene expression among groups of mice were analyzed using the Relative Expression Software Tool (REST). An α -level of 0.05 was used for significance.

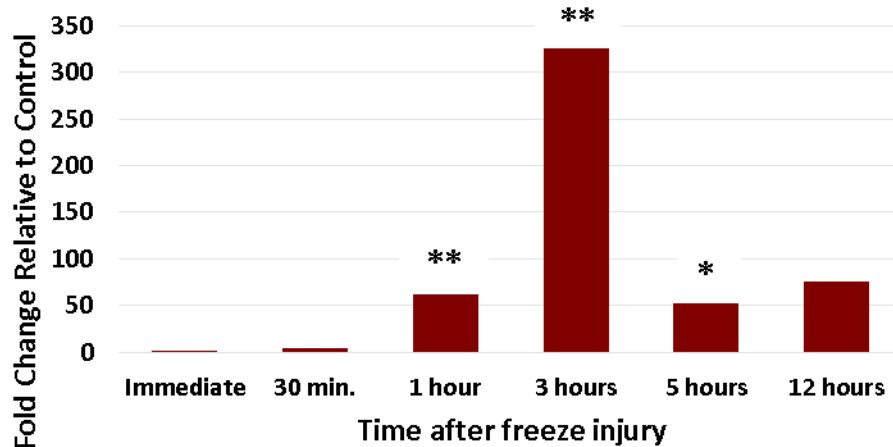
V. Results

Evaluation of pro-inflammatory chemokine expression

Real-time polymerase chain reaction (qPCR) was used to examine how long after freeze injury significant increases in the expression of the pro-inflammatory chemokines CXCL1 and CXCL5 occur, as well as the magnitude of the gene upregulation. When compared to the control group, we found CXCL1 gene expression increases to be highly significant ($p \leq 0.001$) at 1 and 3 hours post-injury and significant ($p \leq 0.05$) at the 5 hour time point (**Figure 1A**). The magnitude of the expression changes was greatest at 3 hours after injury, with a mean expression 325 times greater than the control. CXCL5 expression was increased at 3, 5, and 12 hours after freeze injury (**Figure 1B**). The magnitude of the changes was much larger than in CXCL1, as the mean expression at the 12 hour time point was 23,943 fold greater than the control muscles.

A

CXCL1 Gene Expression

**B**

CXCL5 Gene Expression

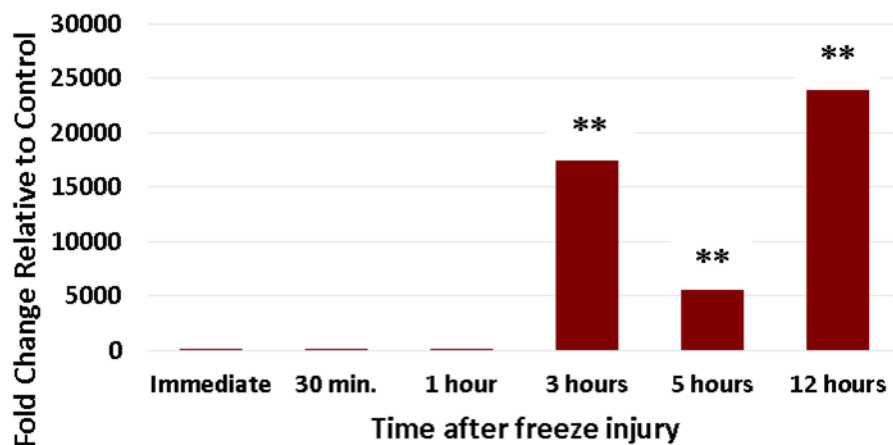


Figure 1- Gene expression changes in inflammatory chemokines begin as early as 1 hour post-injury- Fold changes based on control muscles; * indicates significance ($p \leq 0.05$) between control and freeze-injured muscles and ** indicates high significance ($p \leq 0.001$); $n=3$. A) Chemokine CXCL1; highly significant gene expression changes at 1 hour and 3 hour time points following injury; significant changes at 5 hours post-injury. B) Chemokine CXCL5; highly significant gene expression changes at 3 hours, 5 hours, and 12 hours after freeze-injury.

VI. Discussion

In this study, we showed that traumatic skeletal muscle injury produces a rapid upregulation of CXCL1 and CXCL5 gene expression, beginning as early as 1 hour after injury induction. These early changes are in relative agreement with data from past studies using treadmill running (Nedachi 2008) and electrical pulse stimulation (Nedachi 2009), which first found increases in chemokine-related measures at 30 minutes and 1 hour, respectively. However, it should be noted that CXCL1 and CXCL5 gene expression increases were not found in this study at either the immediate harvest time point or the 30 minute time point. This implies that these pro-inflammatory chemokines are not instantaneously released from the muscle when injured and that there is a time delay between injury and attraction of neutrophils to begin phagocytosis.

The main implication of this finding is that it provides us with clues about just how soon after skeletal muscle injury the immune signal amplification occurs and neutrophils begin to be attracted by pro-inflammatory chemokines to the site of damage. Future directions include increasing the number of mice in each group to reach an adequate sample size for publication, performing fluorescent immunohistochemistry to quantify positive signals and/or assess the localization of the chemokines within the muscle tissue, and running Western blots to identify the time delay between the increase in CXCL1 and CXCL5 gene expression and increased protein levels in the muscle. The Western blot experiments are particularly important, since it is the CXCL1 and CXCL5 proteins, not the mRNAs they are translated from, that attract neutrophils to the site of injury.

VII. Literature Cited

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